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Rapid selection of specific MAP kinase-binders from designed ankyrin repeat protein libraries

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We describe here the rapid selection of specific MAP-kinase binders from a combinatorial library of designed ankyrin repeat proteins (DARPs). A combined *in vitro/in vivo* selection approach, based on ribosome display and the protein fragment complementation assay (PCA), yielded a large number of different binders that are fully functional in the cellular cytoplasm. Ribosome-display selection pools of four successive selection rounds were examined to monitor the enrichment of JNK2-specific DARPs. Surprisingly, only one round of ribosome display with subsequent PCA selection of this pool was necessary to isolate a first specific binder with micromolar affinity. After only two rounds of ribosome-display selection followed by PCA, virtually all DARPs showed JNK2-specific binding, with affinities in the low nanomolar range. The enrichment factor of ribosome display thus approaches 10⁵ per round. In a second set of experiments, similar results were obtained with the kinases JNK1 and p38 as targets. Again, almost all investigated DARPs obtained after two rounds of ribosome display showed specific binding to the targets used, JNK1 or p38. In all three selection experiments the identified DARPs possess very high specificity for the target kinase. Taken together, the combination of ribosome display and PCA selections allowed the identification of large pools of binders at unparalleled speed. Furthermore, DARPs are applicable in intracellular selections and immunoprecipitations from the extract of eukaryotic cells.

Keywords: ribosome display/protein fragment complementation assay/MAP kinase/designed ankyrin repeat proteins (DARPs)/intrabody

Introduction

To understand the function of proteins, they have to be studied in their natural environment. A large number of such experiments depend on reagents for specific molecular recognition. The most commonly used tools are based on antibodies, and they have also been used inside cells in recombinant form ('intrabodies') (Lobato and Rabbitts, 2004). However, recombinant antibody fragments are not ideal for applications in the reducing intracellular environment, as their stability relies on disulfide bonds that do not

form in this milieu. For this reason, antibodies were engineered for higher intracellular stability with some success (Proba *et al.*, 1998; Wörn and Plückthun, 1998; Desiderio *et al.*, 2001; Visintin *et al.*, 2002), but the number of selected and active binders is usually limited (Tanaka and Rabbitts, 2003; Koch *et al.*, 2006). These results can be explained by the selection pressure, which not only places demands on specific binding but also requires stability under reducing conditions.

To overcome these limitations of immunoglobulin domains, a variety of novel scaffolds for the generation of antibody-like binding molecules, some of them possessing very favorable biophysical properties, has been developed (Nygren and Skerra, 2004; Binz *et al.*, 2005). Designed ankyrin repeat proteins (DARPs) represent such a novel scaffold (Binz *et al.*, 2003; Forrer *et al.*, 2003). It has been shown that DARPs match all requirements for functional intracellular applications: these molecules can be selected to bind to any given target protein with high affinity and specificity, they are very well expressed in the bacterial cytoplasm and they are stable and cysteine-free (Kohl *et al.*, 2003; Binz *et al.*, 2004). The selection of DARPs that act as inhibitors of a bacterial kinase demonstrated their intracellular activity (Amstutz *et al.*, 2005). In this experiment, the DARPin was selected to inhibit the activity of aminoglycoside phosphotransferase (3')-IIIa (APH), an enzyme normally mediating kanamycin (kan) resistance, to render the cells kan-sensitive again. The mechanism of action was confirmed by the crystal structure of the complex (Kohl *et al.*, 2005). While the selection of specific *in vitro* inhibitors from other scaffold libraries has also been described (Mathonet and Fastrez, 2004), only few intracellular applications have been reported (Koide *et al.*, 2002).

We report here the rapid selection of large numbers of target-specific DARPs and investigate their intracellular binding-activity, by using a combination of ribosome display (RD) (Hanes and Plückthun, 1997), a selection technology that works entirely *in vitro*, and the protein fragment complementation assay (PCA) (Mössner *et al.*, 2001), an *in vivo* selection technology. Briefly, in RD, a library of mRNA molecules without stop-codon is translated *in vitro*, such that the protein of interest remains connected to the tRNA, and the mRNA also stays connected to the ribosome, thereby linking genotype and phenotype via the ribosome (Hanes and Plückthun, 1997). In the PCA system, two potentially interacting proteins are co-expressed in *Escherichia coli*, each fused to one of the complementary halves of the enzyme dihydrofolate reductase (DHFR). If they interact, they reconstitute the enzyme, allowing the bacterial cell harboring the interacting pair to grow on selective media (Pelletier *et al.*, 1998; 1999; Mössner *et al.*, 2001).

Three members (JNK1, JNK2, and p38) of the mitogen-activated protein kinase (MAPK) family were chosen as

targets. MAPKs are key factors in cellular signal transduction and are involved in a whole range of diseases, including cancer and inflammation (Manning and Davis, 2003; Waetzig and Herdegen, 2005). The investigation of specific MAPKs is challenging, as these enzymes are highly homologous, with highly conserved active sites. This problem becomes even more pronounced for distinguishing members within the different subfamilies. For example, the JNK subfamily (Kallunki *et al.*, 1994) consists of three members, JNK1, JNK2 and JNK3, each with different splice variants (Waetzig and Herdegen, 2005). While JNK1 and JNK2 are expressed ubiquitously, JNK3 expression is mostly restricted to the brain, heart and testis (Gupta *et al.*, 1996). These kinases mediate the activation of cellular responses to extracellular signals (Davis, 2000). JNK-dependent signal transduction is important in a wide range of different cellular processes, including proliferation, oncogenic transformation, programmed cell death and many others. While JNK-specific functions are known, assigning them to the different JNK isoforms is still difficult. For this purpose, binding molecules that can reliably differentiate between these isoforms and are functional in the reducing intracellular environment are needed.

The results obtained with the DARPin library and the combination of RD and PCA demonstrate that a very large number of high-affinity MAPK-specific binders, some of which show high kinase-isoform specificity, were rapidly selected. All the identified binders were functional in the cellular cytoplasm. The results also help explain how RD and PCA complement each other to give an unexpectedly rapid enrichment of binders.

Materials and methods

Molecular biology

Unless stated otherwise, all experiments were performed according to standard protocols (Sambrook *et al.*, 1989). Enzymes and buffers were purchased from New England Biolabs (Beverly, MA, USA) or Fermentas (Vilnius, Lithuania). Oligonucleotides were obtained from Microsynth (Balgach, Switzerland).

Antigen expression and purification

The human MAP kinases JNK1 α 1 (Swiss-Prot accession number P45983-2, MK08_HUMAN, short splice form), JNK2 α 2 (Swiss-Prot accession number P45984-1, MK09_HUMAN, long splice form), and mouse p38 (Swiss-Prot accession number P47811-1, MK14_MOUSE, α -isoform) were used as targets. For some experiments, also the short splice form JNK2 α 1 (Swiss-Prot accession number P45984-2, MK09_HUMAN, short splice form) was used. (An alignment of the sequences is shown in the Supplementary Material available at *PEDS* online). For expression of the antigen constructs the vectors pAT222 (GenBank accession AY327137), pAT222_p38 and pAT222_JNK2 (Binz *et al.*, 2004), as well as pAT222_JNK1 were used. pAT222_JNK1 was constructed by inserting the PCR-amplified JNK1 α 1 open reading frame [oligonucleotides jnk1f 5'-TTC CGC GGA TCC GGT ACC TCC CGT AGC AAG CGT GAC AAC-3' and jnk1r 5'-AAA CCC AAG CTT GCT GCA CCT GTG C-3'; template pAT58, (Forrer and Jaussi, 1998)] into pAT222, using the restriction enzymes *Bam*HI and *Hind*III. Expression from the vectors pAT222, pAT222_p38,

pAT222_JNK1 and pAT222_JNK2 yields a fusion protein comprising an N-terminal avi-tag for *in vivo* biotinylation, bacteriophage lambda protein D (pD), followed by the target molecule (or nothing in the case of pAT222), and a C-terminal His tag for purification (avi-pD-target-His₆). The fusion of a target protein to the well expressed and highly soluble pD may increase the expression yield of the target protein (Forrer and Jaussi, 1998). Alternatively, for expression of His₆-tagged JNK2 α 2 without any further fusion partner, the vector pQE30_JNK2 was used. pQE30_JNK2 was constructed by inserting the *Bam*HI/*Hind*III JNK2 α 2 fragment of pAT222_JNK2 into pQE30 (QIAGEN, Hilden, Germany). For expressions of His₆-tagged short JNK splice variants (JNK1 α 1 and JNK2 α 1) without any further fusion partner, the vector pQE32 (QIAGEN) was used. For construction of the vectors pQE32_JNK1 α 1 and pQE32_JNK2 α 1, the open reading frames of JNK1 α 1 and JNK2 α 1 were amplified by PCR from the templates pAT222_JNK1 and pAT222_JNK2 using the following oligonucleotides: JNK1-Sph-For 5'-GCT CAG GCA TGC TTT CCC GTA GCA AGC GTG AC-3', JNK1-Hind-Back 5'-GAG GAT CCA AGC TTC TAT TAT TGC TGC ACC TGT GCT AAA GG-3', JNK2-Sph-For 5'-GCT CAG GCA TGC TTT CCG ACT CTA AAT GTG ACA G-3', and JNK2-Hind-Back 5'-GAG GAT CCA AGC TTC TAT TAT TGC TGC ATC TGT GCT GAA GGC TGA TC-3', respectively. The amplified PCR products of JNK1 α 1 and JNK2 α 1 were then digested with the restriction enzymes *Sph*I and *Hind*III and finally ligated into the vector pQE32.

Expression, biotinylation and purification of His₆-tagged proteins pD, pD_JNK1 α 1, pD_p38, pD_JNK2 α 2, and non-biotinylated JNK2 (splice variants α 1 and α 2) were performed as described (Binz *et al.*, 2004), whereas non-biotinylated JNK1 α 1 was expressed in BL21/pRep4 cells (QIAGEN) at 25°C. Biotinylation was quantified by using ELISA and SDS-PAGE, followed by blotting with a streptavidin-alkaline phosphatase conjugate (Roche, Basel, Switzerland).

In vitro RD selection

The PCR-amplified DARPin libraries were transcribed, and four selection rounds against the target molecule pD_JNK2 α 2 as well as two selection rounds against the target molecules pD_JNK1 α 1 and pD_p38 were performed as described (Binz *et al.*, 2004). After each RD selection round the RT-PCR product of the encoding DARPin pool was analyzed by agarose gel-electrophoresis. The modular architecture of DARPins can lead to recombination events during PCR leading to shortened DARPins. In this study selections were carried out with a library consisting of three randomized ankyrin repeat modules between an N-terminal and a C-terminal capping repeat (denoted N3C). To prevent an enrichment of molecules which have lost one module (N2C) by recombination during PCR, the N3C pool of each selection round was gel-purified prior to re-amplification of the corresponding DNA. The DNA of each selection round was finally inserted in the PCA selection plasmid and cloned by transformation of *E.coli* XL10 (Stratagene, La Jolla, CA, USA).

In vivo selection using PCA

The RT-PCR products of each RD selection round, as well as the naïve DARPin library, were separately amplified by PCR

using the oligonucleotides Ank-Sph-FOR (5'-GCT CAG GCA TGC TTG ACC TGG GTA AGA AAC TGC-3') and Ank-Eco-BACK (5'-GCT GCA GAA TTC TTG CAG GAT TTC AGC CAG G-3') and inserted via *SphI* and *EcoRI* into the PCA selection plasmid pHK46 (Koch *et al.*, 2006). Transformation of *E. coli* XL10 with the ligation products yielded ~2500 colonies for each of the seven DARPin pools (four JNK2 ribosome-display selection rounds, the naïve DARPin library, the second one JNK1 ribosome-display selection round, and the second one p38 ribosome-display selection round), and virtually no background of re-ligated vector was observed. The colonies of each transformation were pooled separately and the plasmids were isolated.

For cloning of the kinase-mDHFRII antigen constructs the open reading frames of JNK1 α 1 and p38 were amplified by PCR from the templates pAT222_JNK1 and pAT222_p38 using the following oligonucleotides: JNK1-Sph-For (see above), JNK1-Eco-Back 5'-GCT GCA GAA TTC TTG CTG CAC CTG TGC TAA AGG-3', p38-Sph-For 5'-GCT CAG GCA TGC TTT CTC AGG AGC GTC CGA CC-3', and p38-Eco-Back 5'-GCT GCA GAA TTC GGA CTC CAT CTC TTC TTG G-3', respectively. The amplified PCR products of JNK1 α 1 and p38 were then digested with the restriction enzymes *SphI* and *EcoRI* and finally ligated into the vector pHK43 (Koch *et al.*, 2006), yielding the plasmids pHK95 (JNK1) and pHK96 (p38).

For carrying out the *in vivo* PCA selection, electrocompetent *E. coli* BL21/pRep4 (Qiagen, Hilden, Germany) cells (transformation efficiency $\geq 3 \times 10^8/\mu\text{g}$ DNA) were co-transformed in parallel with 100 ng of each DARPin pool-mDHFR and either pHK92 [JNK2 α 2-mDHFRII fusion construct (Koch *et al.*, 2006)] or pHK95 and pHK96. After 1 h incubation at 37°C, the cells were washed with M9 minimal medium and plated on selective M9 minimal media supplemented with 50 $\mu\text{g}/\text{ml}$ kanamycin (kan, lac-repressor encoding plasmid pRep4), 100 $\mu\text{g}/\text{ml}$ ampicillin (amp, DARPin-library encoding plasmid), 10 $\mu\text{g}/\text{ml}$ chloramphenicol (cam, kinase target-encoding plasmid), 2 $\mu\text{g}/\text{ml}$ trimethoprim (TMP, to inhibit bacterial DHFR and select for functional reassembled mDHFR), 100 μM IPTG, and 5 % (w/v) of casamino acids (Difco, Detroit, MI, USA) and incubated at 25°C for 72 h.

In parallel to the selective co-transformation, *E. coli* BL21/pRep4 was transformed with 100 ng of each DARPin pool-mDHFR fusion constructs and incubated overnight at 37°C on standard LB plates (no selection pressure).

ELISA screening to identify specific MAPK-binding DARPins

From the JNK2 selection pools 17 clones each from RD selection rounds 1, 2 and 4, with or without additional PCA selection step (Figures 1 and 2) were randomly picked. For selections on JNK1 and p38, 11 clones from RD selection round 2, again with or without additional PCA selection step (Figure 7), were randomly picked. All selected clones were cultured overnight in 1.2 ml LB (LB, 1% glucose, 100 $\mu\text{g}/\text{ml}$ amp, and 50 $\mu\text{g}/\text{ml}$ kan) in 96-deep-well plates (Abgene, Surrey, UK). On the next day, 300 μl each of these pre-cultures were added to 1.2 ml LB medium, and incubated at 37°C. After 1 h incubation, expression of the DARPin-mDHFR fusion was induced by 0.5 mM IPTG, followed by incubation for 3 h at 37°C. The cultures were harvested by centrifugation and subsequently lysed by shaking for 10 min

in 50 μl B-Per (Pierce, Rockford, IL, USA). After addition of 250 μl TBS₅₀₀ (50 mM Tris-HCl, pH 7.8, 500 mM NaCl) the lysed cells were centrifuged again (4°C). One hundred and fifty microliters supernatant each was transferred to a MaxiSorp plate (Nunc, Roskilde, Denmark), coated with either pD or pD_JNK2 α 2 (for immobilization of pD or pD_JNK2 α 2, see Binz *et al.*, 2004) and either BSA, pD_JNK1 α 1, pD_JNK2 α 2, or pD_p38. After incubation for 45 min at 4°C, DARPin-mDHFR fusion proteins were detected using an anti-RGS(His)₆-antibody as described (Binz *et al.*, 2004).

Expression and purification of selected JNK2-specific DARPins

DNA encoding the selected DARPins were PCR-amplified using oligonucleotides Ank-Sph-FOR and Ank-Hind-BACK (5'-GAG GAT CCA AGC TTC TAT TAT TGC AGG ATT TCA GCC AGG TC-3') and inserted via *SphI* and *HindIII* into the expression vector pQE32 (Qiagen), which is the PCA selection plasmid without the mDHFR fusion protein. Successful cloning was verified by DNA sequencing. Expression and purification of the DARPins was carried out as described (Kohl *et al.*, 2003). Purity of the samples was assessed by SDS-PAGE, and concentrations were determined by UV absorbance at 280 nm.

Specificity of selected JNK2-specific DARPins

The biotinylated proteins JNK2 α 2, JNK1 α 1 and p38 were immobilized on neutravidin-coated MaxiSorp plates (Nunc, Roskilde, Denmark) as described (Binz *et al.*, 2004). One hundred microliters of selected and purified DARPins (250 nM) were incubated in TBST (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 0.05% Tween, and 0.5% BSA) in each well for 30 min at 4°C. In addition, one unselected DARPin (100 μl 250 nM E3_5, see Binz *et al.*, 2003) and two anti-JNK antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a dilution of 1:500 (dilution recommended by the supplier) in TBST served as controls (anti-JNK1 antibody C17/sc-474 and anti-JNK2 antibody N18/sc-827). Furthermore, one aliquot of each binder (DARPin and antibodies) was additionally pre-incubated for 2 h with 750 nM free JNK2 α 2 prior to incubation with the immobilized antigen (Figure 4A). Clone pB4 was applied at higher concentrations (5 μM purified DARPin; inhibited with 15 μM free JNK2 α 2). In the second ELISA (Figure 4B), binding of only the selected DARPins (250 nM) to immobilized JNK2 α 2 was inhibited with free JNK2 α 2, JNK2 α 1 and JNK1 α 1 (750 nM each). Again, DARPin pB4 was applied at higher concentrations (5 μM purified DARPin; inhibited with 15 μM free JNK2 α 2, JNK2 α 1 and JNK1 α 1). In both ELISAs, binding of the selected DARPins was analyzed by detection of their N-terminal RGS(His)₆ motif as described (Binz *et al.*, 2004). Binding of the purchased anti-JNK antibodies was detected by a goat anti-rabbit IgG-AP antibody conjugate [100 $\mu\text{l}/\text{well}$, 1:3000, 45 min; Sigma (St Louis, MO, USA)]. After extensive washing, the bound DARPins as well as the purchased anti-JNK antibodies were visualized using 4-nitrophenyl phosphate (3 mM 4-NPP, 50 mM NaHCO₃, and 50 mM MgCl₂ in H₂O; 100 $\mu\text{l}/\text{well}$). Absorbance was measured at a wavelength of 405 nm. All measurements were performed in duplicates.

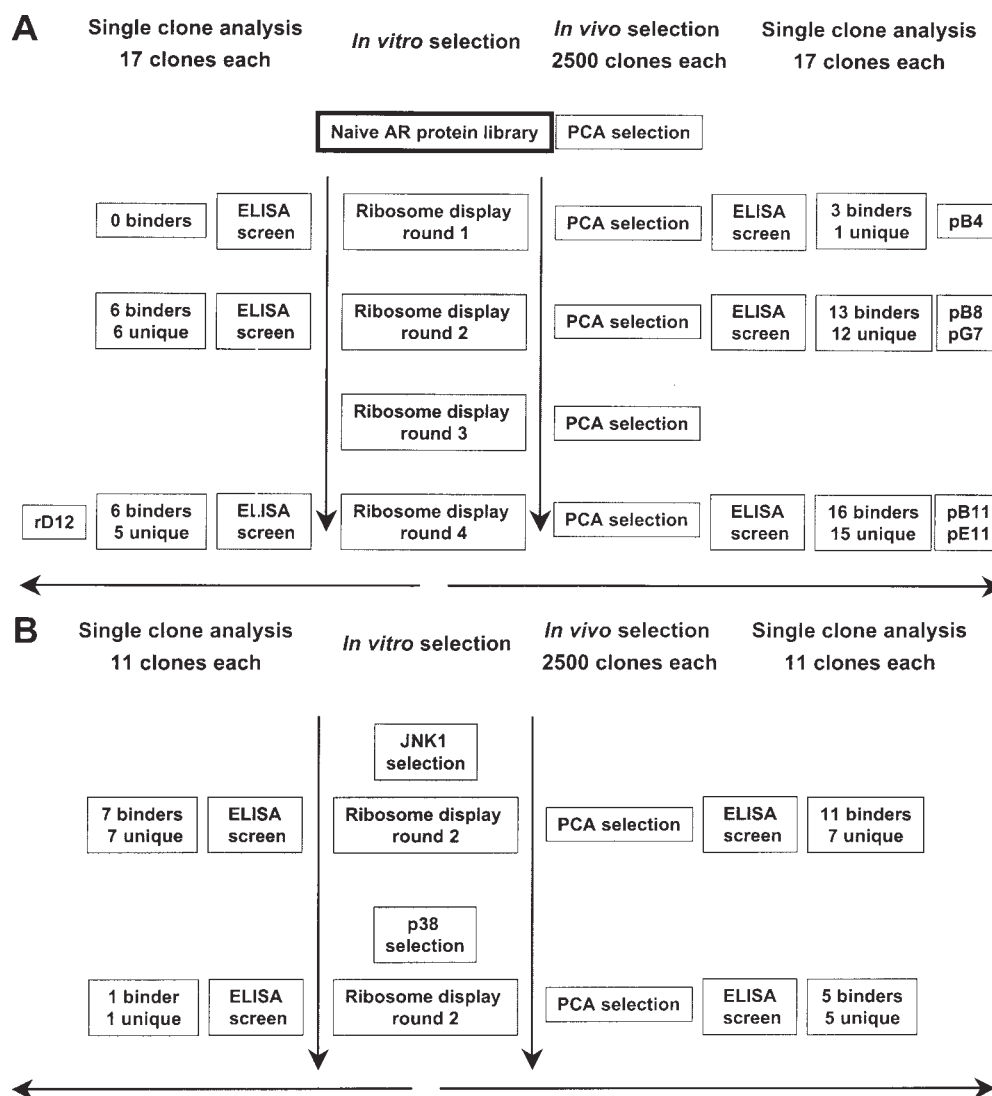


Fig. 1. Schematic representation of the selection and analysis strategy. (A) JNK2 selection. (B) JNK1 and p38 selection.

Surface plasmon resonance

Surface plasmon resonance (SPR) was measured using a BIAcore 3000 instrument (BIAcore, Uppsala, Sweden). All measurements were done in HBS buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 0.005% Tween 20) at a flow rate of 50 µl/min. Biotinylated pD_JNK2α2 fusion protein was immobilized (600 RU) on a SA chip (BIAcore). For the determination of kinetic data, the interactions were measured as follows: 5 min initial buffer flow, followed by a 3 min injection of DARPin in varying concentrations (1–200 nM) and a final off-rate measurement of 45 min with buffer flow. DARPin pB4 was measured at higher concentrations (50 nM to 1 µM) using a shorter dissociation time (15 min). The signal of an uncoated reference cell was always subtracted from the sensorgrams. The kinetic data of the interaction were evaluated with a global fit using BIAevaluation 3.1 (BIAcore).

Affinity precipitation

Human embryo kidney cells (HEK-293T) were lysed in cold lysis buffer (20 mM Tris pH 7.7, 2 mM MgCl₂, 50 mM KCl,

0.1 mM EDTA, 1.0% Triton X-100) and shaken for 10 min at 4°C. The lysate was cleared by centrifugation at 14 000 r.p.m. for 15 min at 4°C. Ni-NTA agarose beads (16 µl of a 50% (v/v) suspension, Qiagen) were washed three times with 500 µl cold lysis buffer. The purified recombinant DARPins (2 µg per assay) were coupled to the beads for 1 h at 4°C. The beads were washed again, and non-specific binding sites of the beads were saturated by incubation with 0.5% BSA for 30 min. Again the beads were washed three times with cold lysis buffer. Five hundred microliters cleared cell lysate per assay (1 mg of total protein) was added to the prepared beads. Binding of DARPins was taking place while tumbling on a rotation wheel at 4°C for 2 h. The beads were washed three times with ice-cold lysis buffer followed by a final wash in TBS. Proteins bound to the beads were eluted in 40 µl elution buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 250 mM imidazole). To the eluate 12.5 µl 4× loading buffer was added and heated to 95°C. Ten microliters of the sample was added per lane and separated by SDS-PAGE on a 10% (for JNK2) or 15% (for ankryrin repeat proteins) gel. As a control for the total cell lysate, 2 µl were loaded. Endogenous JNK2 and the recombinant DARPins were detected by

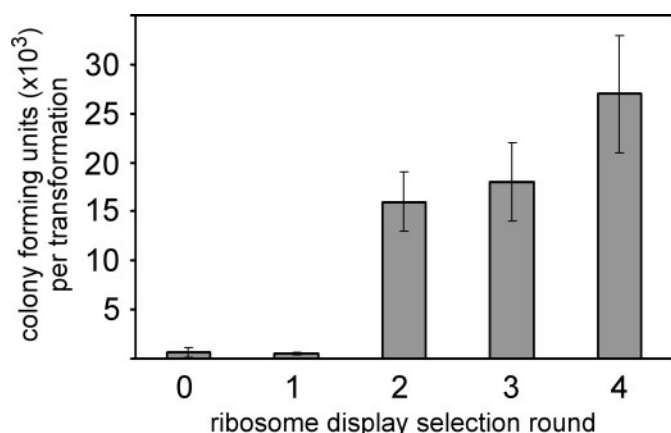


Fig. 2. *In vivo* PCA screen after each JNK2 ribosome-display selection round. The unselected DARPIn library and the DARPIn pools obtained after each round of RD selection were cloned as N-terminal mDHFRI fusion (see Materials and Methods) and co-transformed in *E. coli* with the JNK2 α 2-mDHFRII antigen construct under selective conditions. Co-transformation of the naive DARPIn library or the DARPIn pool of the 1st RD selection round with the JNK2-DHFRII encoding plasmid gave a similar, low level of colony forming units. In contrast, increasing numbers of colonies are observed from co-transformation of the DARPIn pools of RD selection rounds 2, 3 and 4. Under non-selective growth conditions, similar numbers of colonies were obtained.

western blotting. Primary antibodies against SAPK/JNK (Cell Signaling, Catalog No. 9252) and against the RGS-His tag (Qiagen) were used, respectively. Secondary anti-mouse antibodies were visualized by enhanced chemiluminescence (Amersham Biosciences).

Results

The goal of this project was the selection of DARPins binding to MAP kinases with high specificity and to test the functionality of the selected DARPins *in vitro* as well as *in vivo*. At the same time, the selection process was monitored. For initial selections of binding molecules from large DARPIn libraries, RD (Hanes and Plückthun, 1997) was applied. This complete *in vitro* selection technology circumvents any library-size limiting transformation step and is thus ideally suited to handle large libraries (diversity $\geq 10^{10}$). PCA, a bacterial intracellular selection technology, based on the reconstitution of DHFR activity from the divided enzyme by two interacting partners (Pelletier *et al.*, 1998, 1999; Mössner *et al.*, 2001), was subsequently applied to analyze the RD generated pools of binders (Figure 1) and to further enrich these binders. This assay, taking place in the bacterial cytoplasm, also demonstrates DARPIn activity under reducing conditions. Selected DARPins were further examined for their target binding properties with respect to specificity and affinity in ELISA, SPR, and affinity precipitation experiments.

In vitro and *in vivo* selection of JNK2-binding DARPins

For the selection of JNK2-specific binders from an N3C DARPIn library, four standard RD selection rounds (Hanes and Plückthun, 1997) against immobilized JNK2 α 2 (long splice variant of JNK2) were performed (for an explanation of the nomenclature N3C, see Materials and Methods). The alignment of the short and long splice variants of the MAP kinases used in this study are shown in the Supplementary Material available at PEDS online. A pool of DNA fragments encoding DARPins (~2500 individual clones) from the naive

starting library and from each RD selection round was inserted into the PCA selection plasmid as a mDHFRI fusion. In parallel experiments, co-transformation of these pools with the JNK2 α 2-mDHFRII-bait-plasmid allowed determination of the bacterial survival under selective conditions as a function of the respective RD selection round (Figure 2).

As expected, nearly no bacterial colonies resulted from co-transformation of the bait-plasmid with the naive DARPIn library. The pool after the first round of RD also gave rise to almost no colonies under selective PCA conditions (i.e. in the presence of TMP to inhibit bacterial DHFR). In contrast, co-transformations of the DARPIn pools corresponding to RD selection rounds 2, 3 and 4 gave rise to a large number of bacterial survivors, which increased with the selection round number (Figure 2). As a control, the individual pools were also plated under non-selective conditions with no TMP present, where no difference in colony numbers was observed, indicating that all pools had been treated equally (data not shown). These results suggest that two rounds of RD are sufficient for selection of binders.

ELISA screen to identify JNK2-specific DARPins

To elucidate whether the increased colony number, observed in the *in vivo* PCA selection, did indeed result from an enrichment of JNK2-specific DARPins, the binding of the selected DARPins to JNK2 α 2 was tested in ELISA screens. Seventeen clones from each of the selection rounds 1, 2 and 4, obtained either after growth on selective media for PCA selection or from colonies grown on non-selective plates, were randomly picked. Crude *E. coli* extracts from small-scale expression cultures were used in ELISA experiments to compare the binding of the selected clones either to JNK2 α 2 or, as a control, to the pD (Figure 3).

Of the DARPins, which were selected by RD without subsequent PCA step, no JNK2-specific DARPIn was found in the pool from selection round 1, whereas ~30% of the DARPins of both the selection rounds 2 and 4 showed JNK2-binding. In comparison, when a PCA selection was carried out after the first RD selection round, one JNK2 binder was already obtained. Furthermore, ~80% of the DARPins of round 2 and nearly 100% of the DARPins of round 4 showed binding to JNK2, when the RD rounds were followed by a PCA selection. It seems that RD is afflicted with an intrinsic background, which cannot be reduced by further RD selection rounds. Thus, the expression plasmids which were obtained by ligation of the RD product, encode a constant percentage of non-binders. Typical values of 10–70% non-binders (see also below for the second set of experiments) have been found in other experiments. In contrast, when the same pools are sieved by an additional PCA selection, already after the second round of RD, the majority of clones are specific binders. PCA alone, on the other hand, suffers from a background of false positives, if selections are carried out with naive libraries (Koch *et al.*, 2006).

We have thus used PCA as a tool to analyze the progress of the RD selection from round to round. The enrichment in RD is much faster than previously assumed. However, it does not reach a level of 100% binders by itself: the errors introduced by PCR, point mutations and recombination events (see below), that make RD so attractive as an evolution technology also cause a constant background of non-binders. On the other hand, we can use PCA also as an

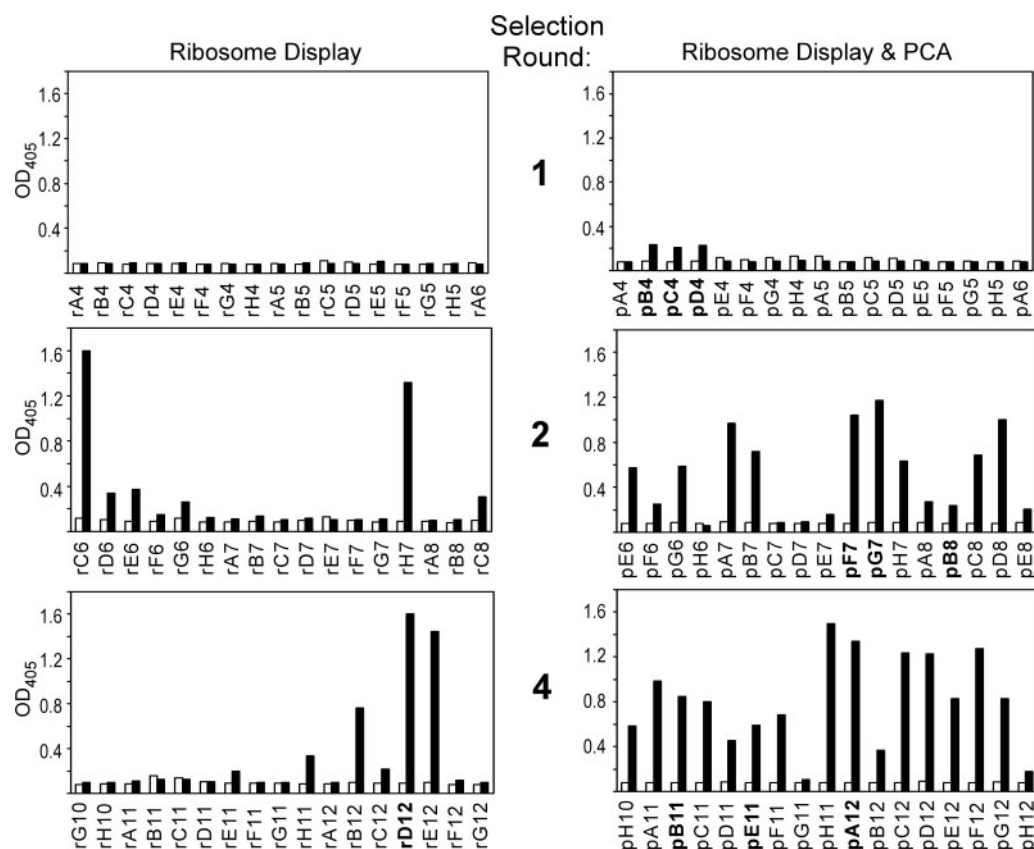


Fig. 3. ELISA screen to identify JNK2-specific DARPins. The JNK2-binding of randomly picked DARPins of different RD selection rounds (with or without additional PCA selection step) are compared. Crude cell extracts of *E.coli* expressing the DARPins as mDHFRI fusions were applied to wells coated with JNK2 α 2 (black columns) or pD (control; white columns). No JNK2-specific DARPins were identified from selection round 1 of the RD selection, whereas three JNK2-specific DARPins (determined to have the identical sequence) were found in round 1, when followed by an additional PCA selection. From the groups of DARPins originating from RD selection rounds 2 and 4, ~30% possess JNK2 specificity. Again, many more JNK2-specific DARPins were identified from the corresponding selection rounds, when an additional PCA selection was carried out: ~80% of the DARPins of round 2, and nearly all DARPins of round 4 show specific binding to JNK2. All the binders, which were chosen for further characterization, are marked bold (pB4 = pC4 = pD4; rD12 = pF7 = pA12). It can be seen that the performance of the additional PCA screen leads to a much stronger enrichment of JNK2-specific DARPins in all selection rounds evaluated.

integral part of the selection procedure. As the RD-selected pool must be cloned anyway by ligation of the PCR product and transformation of bacteria, the workload remains identical, just a different vector and selective media for plating the bacteria is used.

Sequencing of the JNK2-specific DARPins

Thirty-four DARPins were chosen for sequencing (10 binders from the RD-only selection and 24 binders from the combined RD/PCA selection). Three binders were found multiple times, but only in the selection that combined RD with PCA (pB4 = pC4 = pD4, pF6 = pG6, pF7 = pA12; Figure 3). In addition, two binders were found in either selection (pE8 = rC6, pF7 = pA12 = rD12).

Despite the occurrence of duplicate clones, the total number of different binders found after each round of RD is somewhat larger with the additional PCA step. The main reason is that, in the absence of PCA, a constant fraction of non-binders are found, while in the presence of PCA, the non-binders have all been removed. We have not detected any notable feature that would explain multiple appearance of certain clones (affinity, solubility or expression yield) and cannot exclude a mere statistical effect.

Of the non-binder population, 13 clones without and 13 clones with additional PCA selection were sequenced. In

this group, no deletions, premature stop codons or frame shifts were detected. However, a substantial variation of DARPins sizes, ranging from one (N1C) to five (N5C) internal repeats was found among the non-functional molecules. This finding strongly suggests that recombination events, as discussed above, had taken place during RT-PCR. The power of selection for specific binding keeps the functional molecules to an appropriate size however. Note that this RT-PCR is necessary after the final isolation of mRNA in RD before ligating it into a plasmid which is in turn necessary for the characterization of single clones, isolated from bacteria.

When monitoring the outcome of the RT-PCR after a RD selection round by agarose gel-electrophoresis, a certain proportion of the DARPins were found to be shortened by one AR module (N2C instead of N3C). This shortening was found for 10% of the RD selected DARPins, but, surprisingly, for 50% of the DARPins resulting from the subsequent PCA screen. This observation might indicate a growth advantage of the N2C DARPins over the N3C molecules in the context of the JNK2 PCA selection. This phenomenon has, however, not been observed in the second set of selections (see below).

In conclusion, from 34 sequenced JNK2-binding DARPins, 28 different DARPins were identified, indicating that the generated binder pool still holds substantial diversity.

Table I. Affinity of selected JNK2-specific DARPinS^a

| Clone | Round | Selection | Size | k_{on} ($M^{-1}s^{-1}$) | k_{off} (s^{-1}) | K_D (nM) |
|-------|-------|------------|------|-----------------------------|--------------------------------|----------------|
| pB4 | 1 | RD and PCA | N2C | $(4.1 \pm 2.2) \times 10^4$ | 0.0342 ± 0.0041 | 914 ± 568 |
| pB8 | 2 | RD and PCA | N2C | $(1.4 \pm 0.6) \times 10^5$ | $(9.2 \pm 2.9) \times 10^{-4}$ | 6.5 ± 4.3 |
| pG7 | 2 | RD and PCA | N3C | $(3.5 \pm 1.3) \times 10^5$ | $(4.0 \pm 0.6) \times 10^{-3}$ | 11.3 ± 5.9 |
| pB11 | 4 | RD and PCA | N3C | $(2.0 \pm 0.5) \times 10^5$ | $(4.0 \pm 0.6) \times 10^{-3}$ | 22.0 ± 9.8 |
| pE11 | 4 | RD and PCA | N2C | $(4.2 \pm 1.5) \times 10^5$ | $(9.6 \pm 0.7) \times 10^{-4}$ | 2.3 ± 1.0 |
| rD12 | 4 | RD | N2C | $(4.7 \pm 0.2) \times 10^5$ | $(6.8 \pm 0.9) \times 10^{-4}$ | 1.4 ± 0.3 |

^aKinetic SPR measurements. The global fits can be found in the supplementary data of PEDS online.

Affinity of selected JNK2-specific DARPinS

Target binding of six different JNK2-binding DARPinS was characterized by SPR and by ELISA experiments. Binders from the different selection rounds, with and without additional PCA-selection step, were included (Figure 3).

Kinetic SPR measurements on a Biacore instrument using multiple concentrations of the selected DARPinS were used to determine their dissociation constants with a global fit (Table I). (The global fits are shown in the Supplementary Material available at PEDS online). While the K_D of pB4, the only clone that resulted from the first selection round, was around 1 μ M, all other binders had low nanomolar affinities. Interestingly, the affinity of binders from selection round 2 already equal the ones found from selection round 4. Binders generated with or without additional PCA selection seemed to bind with similar affinities (Table I). As PCA yielded binders possessing affinities ranging from the micromolar (first round) to the nanomolar range (subsequent rounds), it seems that the PCA selection system can tolerate a broad spectrum of affinities, all resulting in bacterial growth. PCA functions thus as a qualitative ‘gate’ in this system (see Discussion).

Specificity of selected JNK2-specific DARPinS

ELISA experiments were carried out to investigate the specificity of the JNK2 binders. In a first ELISA experiment the binding of the DARPinS to JNK2 α 2 (long splice variant) was compared with that to JNK1 α 1 (short splice variant) and p38 (for an alignment of the three kinases and their splice forms, see Supplementary Material available at PEDS online). While JNK2 and JNK1 share 81% identity and 86% similarity, JNK2 and p38 still share 51% identity and 59% similarity on the amino acid level. As a control, two commercially available antibodies (see Materials and Methods), one raised against JNK1, the other against JNK2, were tested in parallel. All DARPinS and the JNK1 antibody were highly specific for their cognate antigen, while the anti-JNK2 antibody seemed to not discriminate between JNK1 and JNK2 (Figure 4). None of the binding proteins interacted with p38.

To investigate the isoform specificity of the selected DARPinS, competition ELISAs were performed. For this purpose JNK2 α 2 (long splice variant) was immobilized, and the binding of the DARPinS was competed with free JNK2 α 2, JNK2 α 1 (short splice variant) or JNK1 α 1 (short splice variant). As expected, the binding of all DARPinS was inhibited by JNK2 α 2, which was the target used for selection. Most DARPinS (pG7, pB8, pB11, pE11) were also inhibited by JNK2 α 1. While the inhibition of DARPin pE11 was equal for both long and short isoforms of JNK2, the others (pG7, pB8, pB11) were inhibited less by the short JNK2 splice

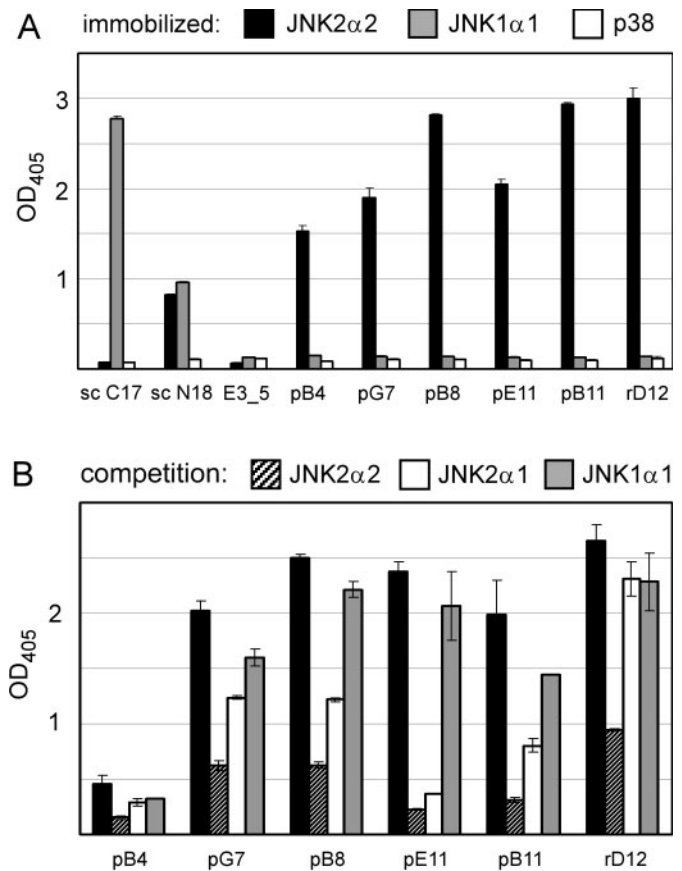


Fig. 4. Specificity comparison of JNK2-selected DARPinS by ELISA. (A) Binding of different selected DARPinS to JNK2 α 2, JNK1 α 1 and p38 is compared. Two commercially available antibodies (sc C17 and sc N18, see Materials and Methods) and one unselected DARPin (E3_5) were included as controls. Because of its lower affinity DARPin pB4 (obtained after only one round of RD) was applied at a concentration of 5 μ M, compared with 250 nM for all other DARPinS (A and B). All selected DARPinS and the JNK1 antibody (sc C17) were highly specific for their respective antigen in immobilized form, while the anti-JNK2 antibody (sc N18) does not discriminate between the JNK homologs. The unselected DARPin (E3_5) does not bind to any of the antigens. Furthermore, none of the tested binding proteins show an interaction with p38. (B) A competition ELISA including the different JNK splice variants was used to evaluate the specificity. The black bars indicate the signal in the absence of competitor. All binders were inhibited by a 3-fold excess of free JNK2 α 2 (final concentration 750 nM). The same amount of JNK α 1 had only a minor inhibitory effect on JNK α 2 binding. DARPin pE11 was equally well inhibited by the long and the short splice variant of JNK2, while not inhibited by JNK1, indicating a very high specificity.

variant. This might be explained by the binding epitope residing partly on the extension that characterizes the long isoform, or, alternatively, by the two splice variants being present in two different conformations, of which the one of JNK2 α 2 is bound with higher affinity. All DARPinS were not or only weakly inhibited by the short splice variant of JNK1

(JNK1 α 1). Thus, the DARPins recognize various features discriminating long and short splice variants and JNK1 α 1 and JNK2 α 1. Furthermore, pE11 discriminates JNK1 and JNK2 both in direct binding and competition ELISA.

JNK affinity precipitation with selected JNK2-specific DARPins

In order to test whether the selected DARPins bind to JNK in eukaryotic cells, some of the JNK2-specific binders were tested in affinity precipitation experiments from different cell lines. Lysed cells were incubated with His-tagged DARPins, which were subsequently captured by Ni-NTA beads. The affinity precipitated JNK was detected on western blots with JNK-specific antibodies. All the tested DARPins (pG7, pE11, rD12) precipitated JNK from the cell lysate, giving rise to the corresponding bands on the western blot, while the controls, the non-binding DARPins E3_5 and beads with no DARPins, did not (Figure 5). Both short and long splice variants were precipitated, while the short form predominated. This most likely reflects the ratio of the JNK variants present in the cell lysate. From other cell lines, the large variant was predominately detected by this affinity precipitation and western blot (data not shown). In all tests, the three JNK2-binding tested DARPins gave the same results with respect to the abundance of long and short splice variants. This was not expected from the ELISA experiments, where pG7 and rD12 showed preferences towards the long variant, while pE11 bound both with equal affinity, but we do not know the stoichiometries in the experiment. The affinity precipitation

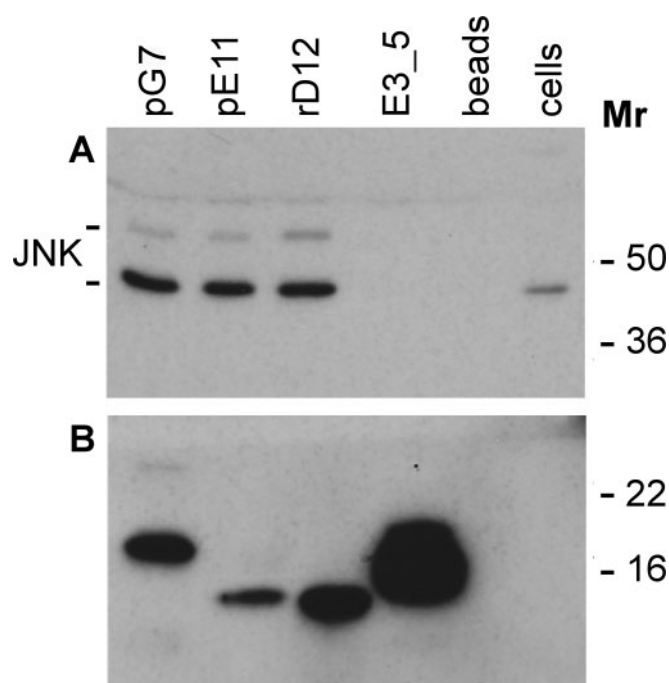


Fig. 5. Affinity precipitation. (A) JNK was immunoprecipitated from human embryo kidney (HEK) cells by selected JNK2-binding DARPins and detected by western blot using a JNK-specific antibody. All tested DARPins (pG7, pE11, rD12) lead to detection of bands on the blot that correspond to JNK, while the controls, non-binding DARPins E3_5 and beads with no DARPins, did not. Both short and long splice variants were precipitated. The MW of the splice forms of JNK2 are 46 and 54 kDa (see Supplementary Material available at *PEDS* online). With respect to specificity for JNK1 or JNK2 in the affinity precipitation experiments no conclusions can be drawn, as the detection antibody does not allow differentiating between the JNK homologs. (B) The presence of the DARPins was confirmed by western blotting (detection of the his-tag).

experiments do not allow any conclusions with regard to specificity toward JNK1 and JNK2, as the detection antibody does not allow differentiating between the different JNK homologs.

Selection and characterization of JNK1- and p38-binding DARPins

To elucidate whether two rounds of RD are sufficient in general to obtain antigen-specific DARPins when cloning of the RD-pools is followed by subsequent PCA selection, additional RD selections against immobilized JNK1 α 1 and immobilized p38 were carried out. Again, the pools of DNA fragments encoding ~2500 individual DARPins obtained after the second round of RD were inserted into the PCA selection plasmid as mDHFRI fusion. The pools (including the JNK2 pool from the first set of experiments) were co-transformed with the cognate kinase and the respective other two kinases as mDHFRII-fusions. As can be seen in Figure 6, the DARPins pools selected against JNK1 and p38 are highly specific for their cognate target kinase, while the JNK2-selected DARPins pool shows some cross-reactivity with JNK1.

Eleven clones from each of the JNK1 and p38 selections obtained either with additional PCA selection or from colonies grown on non-selective plates were randomly picked to inoculate small-scale expression cultures. Crude *E. coli* extracts from these expression cultures were then used in ELISA experiments to compare the binding of the selected clones either to BSA, JNK1 α 1, JNK2 α 2 or p38 (Figure 7). Of the DARPins which were exclusively selected by RD, ~60% of the JNK1-selected clones showed JNK1 binding, while only 10% of the p38-selected clones bound to p38. In comparison, when a subsequent PCA selection was carried out, the proportion of identified binders increased to 100% for the JNK1-selected clones and to 50% for the p38-selected clones on their respective cognate antigen. Thus, for all investigated selection experiments the percentage of binders was increased when the RD rounds were followed by a PCA selection. Furthermore, with the exception for one clone

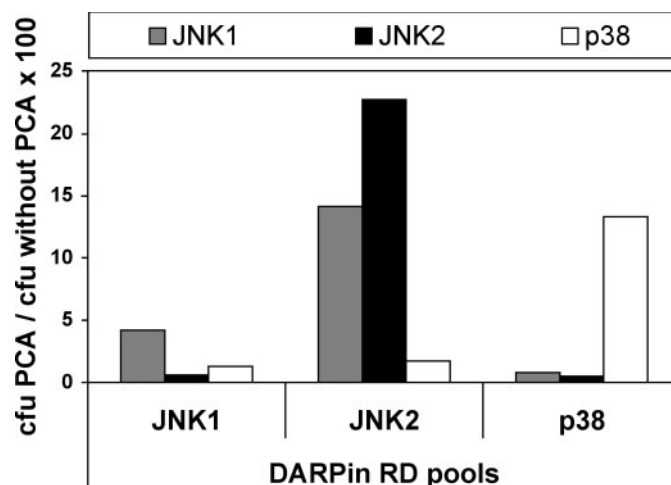


Fig. 6. Co-transformation of the RD-selected DARPins pools (2nd RD selection round) with their respective cognate target kinase and two unrelated kinases. All co-transformations were plated on selection media containing 0.1 mM IPTG and incubated for 72 h at 25°C. The DARPins pools selected against JNK1 and p38 can be seen to be highly specific for their cognate target kinase, while the JNK2-selected DARPins pool shows some cross-reactivity with JNK1.

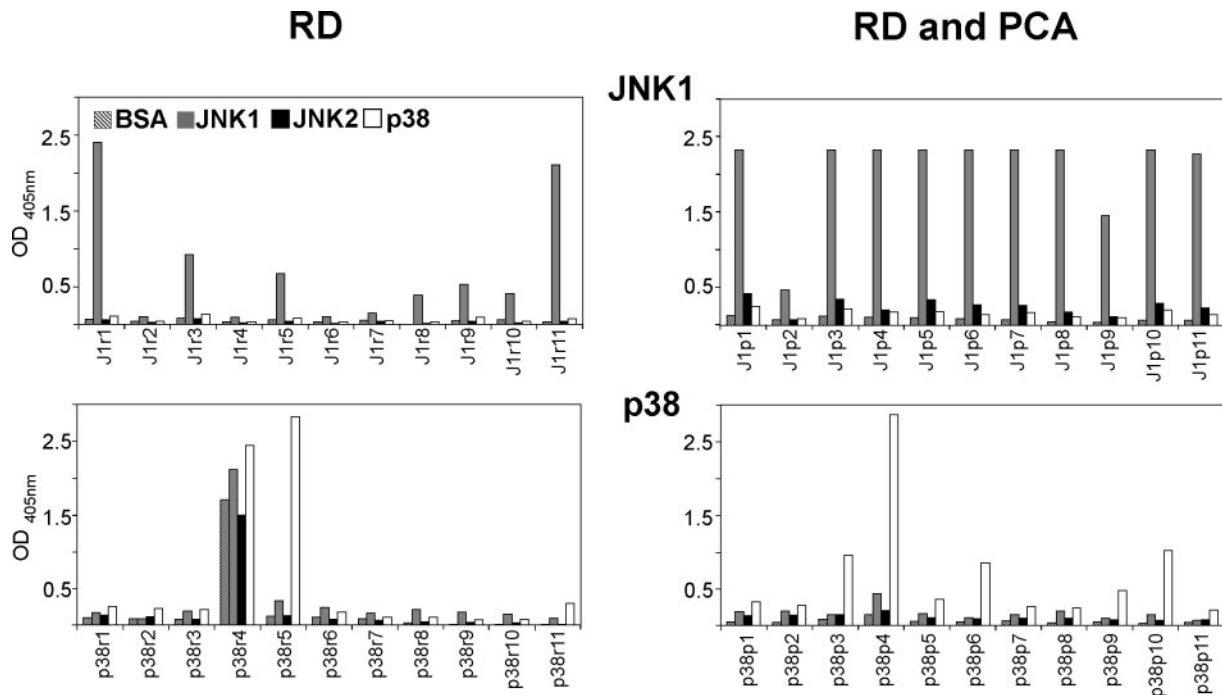


Fig. 7. ELISA screen to identify JNK1- and p38-specific DARPins. The binding of randomly picked DARPins of the 2nd RD selection round obtained from selections against either JNK1 or p38 (with or without additional PCA selection step) to different MAP kinases is compared. Crude cell extracts of *E. coli* expressing the DARPins as mDHFR1 fusions were applied to wells coated with BSA or JNK1 α 1, JNK2 α 2, and p38. From the groups of DARPins originating from only RD selection, ~60% of the constructs show binding to JNK1 whereas only 10% of the clones show binding to p38. In both experiments a higher percentage of cognate DARPins, recognizing JNK1 (100%) or p38 (50%) were identified when an additional PCA selection was carried out. With exception of the clone p38r4 all identified binders show highly specific binding to only the MAP-kinase they have been selected against.

(p38r4), all antigen-binding DARPins were highly specific for the respective target kinase, and did not show binding to the other immobilized members of the MAP-kinase family (Figure 7).

From the group of JNK1-specific DARPins, which were identified in the ELISA screen, five DARPins resulting from RD only and eleven DARPins obtained from the combined RD/PCA selection were chosen for DNA sequencing. From the five JNK1 binders that were selected only by RD none was found multiple times, whereas two of the DARPins that were identified after additional PCA selection were found multiple times (J1p3 = J1p7 and J1p1 = J1p8 = J1p10 = J1p11). None of the six sequenced p38-binding DARPins was found multiple times. (For sequences of the selected DARPins, see Supplementary Material available at *PEDS* online).

In contrast to the sequencing results of the JNK2-specific DARPins, none of the selected p38 binders and only one of the JNK1 binders (J1r5) was shortened by one AR module (N2C instead of N3C).

As 18 of the 22 sequenced JNK1- and p38-binding DARPins are different, the generated binder pools hold again a substantial diversity. Thus, our results indicate that two rounds of RD are indeed sufficient to select highly specific and highly diverse DARPin pools.

Discussion

We demonstrate here the selection of a large pool of DARPins binding to MAP kinases with high specificity and affinities. For the generation of kinase-specific DARPins a combined *in vitro* and *in vivo* selection was performed. RD was applied for the selection of kinase binders from a large combinatorial DARPin library. PCA was used to monitor the

RD selection process and to further enrich specific DARPins from the pools of binders generated by RD. This assay also served to test the functionality of the selected DARPins under reducing conditions in the bacterial cytoplasm. Affinity precipitation from mammalian cell lysates demonstrated another application of DARPins.

The analysis of the binders from different selection rounds showed that the selection process of kinase-specific DARPins was extremely rapid. In three independent selection experiments two rounds of RD were sufficient for enrichment of a variety of different kinase-specific DARPins with nanomolar affinities. These results suggest that the combination of the DARPin library with both RD and PCA selections is very suitable to rapidly generate diverse pools of specific binders, fully functional under reducing conditions. The pools can then be used as starting points to screen for functionality for a range of different applications, including intracellular ones.

Targeting kinases—an issue of specificity

About 518 human protein kinases are currently known (Manning *et al.*, 2002). Most of these are part of complex signaling cascades, and the different kinases often share very high homology. This homology complicates the selection of specific eukaryotic protein kinase (EPK) binders. To address EPK function, often binders and inhibitors are used which lack sufficient specificity, and thus the observed effects cannot be allocated correctly. All selected DARPin binders reported here were highly target kinase-specific. One binder (pE11) seemed to discriminate between JNK1 and JNK2 in direct and in inhibition ELISA, despite 80% identity of the

target proteins. This high specificity of DARPins may be a useful feature in the quest to understand the function of individual kinases in more detail.

We believe that the high specificity of the selected DARPins is based on two components: the binding epitope and the binding mode. Most peptides and small molecules used to date bind EPKs in grooves (Noble *et al.*, 2004). These grooves, especially the active site, are often highly conserved, preventing specific targeting. As the randomized DARPins surface is comparatively flat, the selected binders will interact with the target protein on exposed surface areas. These surface areas are more likely to be kinase-specific, and thus specificity is achieved more easily. From the crystal structures of DARPins library members, unselected or in complex with the target (Kohl *et al.*, 2003; Binz *et al.*, 2004), we know that the DARPins behave essentially like a rigid body and does not bind to grooves or pockets on the targets, but rather to surface structures. This might restrict the compatible binding epitopes, but results in very high specificity, as the DARPins will not adapt to conformations of other proteins.

Selection by a combination of RD and PCA

We have used a combination of RD and PCA, in which PCA was used, on the one hand, as an analytical tool to monitor the progress of RD, and on the other hand, as an additional 'gate' to increase the proportion of binders. We found that one round of RD can be enough to find some binders, if the RD selection is followed by a PCA 'gate'. Because of the sparse sampling at this point (see below), this binder has only micromolar affinity. It takes only one additional round of RD alone, however, to obtain binders of low nanomolar affinity. If RD is thus combined with the PCA 'gate', then all binders may already have these properties, and there is no discernable improvement of affinities from round 2 to round 4.

This surprisingly rapid selection also guarantees the maintenance of high diversity: of 56 sequenced binders, 46 were different. In selections of antibody libraries with RD alone, more rounds were necessary, and then a decrease of diversity became noticeable (Hanes *et al.*, 2000). Clearly, the high diversity of high-affinity binders is an important prerequisite to use them as starting points for selection for function. The selection of DARPins inhibiting a prokaryotic kinase, which is structurally homologous to MAP kinases, has recently been carried out (Amstutz *et al.*, 2005) and the inhibition mechanism was visualized by the structure determination of the complex (Kohl *et al.*, 2005).

The main functioning of the additional PCA step is that of a 'gate'. The PCA selection requires only a micromolar interaction (K_D of pB4, Table I and Figure 3), but it rather effectively removes those clones which do not express binders (Figures 3 and 7). Such non-binders can end up in a selected plasmid by two mechanisms. First, mRNA encoding non-binders can still be present in the target-bound fraction after RD. Second, new non-binders can be generated *via* mutation in the RT-PCR following mRNA isolation, as this PCR step is necessary for cloning. We presume that the latter is an important mechanism contributing to non-binders as it would explain why one cannot obtain 100% binders with additional rounds. We would stress that this finding is not an impediment of the RD method: a great diversity of

clones with high affinity and specificity is always found, and the final percentage of binders is high enough that ELISA screening is very convenient.

The gating function of PCA is illustrated in the outcome of the first round of RD. Binders are rare among the DARPins, as expected from a diverse library, and it is the large size of the library that allows the selection of such binders. PCA alone does not allow the easy identification of such binders, as non-specific interactions occur *in vivo* that lead to cell growth of the naïve library and after one round of RD (Figure 3). Importantly, such non-specific binders are also extremely rare, as the very small number of colony forming units show. As soon as RD has enriched 'real' binders above this background, PCA can be used to reliably identify binders, as by then, background clones are so rare among growing colonies that they are negligible. This 'cross-over' point seems to be reached already after one round of RD. The binders obtained at this stage are not yet of high affinity. The most likely explanation is a statistical one: the sampling of binders is so sparse at this stage that it is likely that anyone that is found will be of low affinity, of which there will be a greater number than those with high affinity. Since only a small subset of the large RD library was cloned, statistically not many binders can be expected. Importantly, it takes only one additional round of RD, until the identified binders are all of low nanomolar affinity (Table I).

We believe that RD for selecting large pools of binders in combination with PCA for sieving these pools is an ideal combination for rapid identification of many different binders. This combination of *in vitro* selection (by phage display) and a subsequent *in vivo* screen (by the yeast two-hybrid system) has also been described for the selection of antibody fragments active inside cells (Visintin *et al.*, 2002; Tanaka and Rabbitts, 2003). By this combination, only very stable antibodies survived selection in the reducing intracellular environment, and many binders might be lost due to insufficient stability. DARPins are stable *per se* under reducing conditions and therefore, probably no binders will be lost in the second *in vivo* selection step. Instead, the selected binder pool is 'cleaned up' from PCR errors by PCA, and at the same time, non-specific *in vivo* interactions no longer play a significant role in PCA, as enriched binders outnumber them.

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